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Role of platelets in early pathogenesis of viridans group streptococcal endocarditis

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In the pathogenesis of infective endocarditis the adherence of circulating bacteria to a preexisting cardiac vegetation (VG) and the survival of bacteria adherent to VGs are major events. One objective of the study was to determine whether the capacity of various Streptococcus sanguis type II strains to adhere to VGs coincided with the ability of the strains to produce endocarditis in rabbits with left heart catheter-induced vegetations (Chapter II). A significant difference in the frequency of culture-positive VGs between two test strains was observed when low concentrations of the bacteria were injected. The incidence of endocarditis caused by one of the test strains (strain 1) was significantly lower than the incidence of culture-positive VGs at 5 min after challenge. It appeared that adherent bacteria of strain 1 to VGs disappeared quite rapidly with time. Disappearance was neither due to detachment of bacteria nor to the complement-dependent serum bactericidal system. Phagocytosis of attached bacteria, as demonstrated in in vitro studies did not occur.

Since platelets accumulate at the vegetational surface after bacterial deposition, further studies were performed whether platelets attributed to the mechanism by which adherent bacteria on the cardiac VGs disappeared early after deposition (Chapter III). In vitro studies showed that washed rabbit platelets stimulated by thrombin released platelet-associated bactericidal substances (PABS). PABS displayed a rapid bactericidal effect on strain 1, but slightly affected the viability of the other strain used in this study, strain 2.

Since it has been reported that in rabbits sub-inhibitory serum levels of streptomycin prevented the development of enterococcal endocarditis, we treated rabbits with a low dose of streptomycin, yielding sub-inhibitory serum levels of streptomycin, and injected them with ID₉₀ inocula of strain 1. None of these rabbits developed endocarditis. The frequency of infection due to strain 2 in streptomycin treated rabbits was not different from control rabbits. In vitro tests revealed that the killing effect by PABS in combination with sub-inhibitory concentrations of streptomycin on the PABS-susceptible strain 1 was enhanced, but not on the PABS-resistant strain 2. Therefore, the selectivity of the interactions of PABS with the strains observed in vitro may well explain the results noted in vivo.

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PABS prepared from rabbit and human platelets as well as the release by thrombin stimulation were described (Chapter IV). Supernatants of sonicated or thrombin-stimulated platelets exhibited antibacterial activity against a limited range of Gram-positive bacteria. Bacillus subtilis strains and a Streptococcus pneumoniae strain rapidly lost viability followed by lysis. S.sanguis type II and two other streptococcal species were killed without lysis. Viability of Gram-negative bacteria, so far tested was not affected. Antibacterial activity was best expressed at acidic condition (pH 5). Bacterial killing was independent of the presence of calcium, complement, and lysozyme. Lysosomal platelet enzymes were not present in the supernatants. SDS-PAGE analysis and iso-electric focussing revealed that the bactericidal activity was confined to low-molecular weight (ca. 2,000 - 6,000 dalton) proteins with a high cathodal migration. Short stimulation with low amounts of thrombin released these proteins from platelets. We have introduced a new term for PABS: thrombodefensins. This term indicates that the presence of thrombin is a prerequisite and that the proteins exhibit antibacterial activity.

Since it is thought that bacteria deposited onto the cardiac vegetations induce platelet aggregation, it seemed of interest to investigate whether or not bacteria-induced platelet aggregates released thrombodefensins (Chapter V). Platelet aggregation was induced by S.sanguis type II and two platelet agonists, ADP and thrombin alone or in combination. The platelet release reaction by assaying serotonin, lactate-dehydrogenase and thrombodefensins, was evaluated to gain insight into platelet storage sites of thrombodefensins. Bacteria- or ADP-induced platelet aggregates showed a minimal release of thrombodefensins. Bacteria sequestered within these aggregates remained viable. In response to sequential treatment by thrombin, the bacteria- or ADP-induced aggregates rapidly released thrombodefensins. Indomethacin a cyclooxygenase inhibitor, prevented almost completely the release of thrombodefensins from platelets stimulated with very low concentrations of thrombin. Most probably platelet α -granules are storage sites for thrombodefensins.

In platelet clots bacteria lost markedly viability. Apparently, a cooperative interaction between platelet-released thrombodefensins and acid lysosomal enzymes exists, since these enzymes lower the pH level

within clots.

The response of S.pneumoniae and S.sanguis type I and type II strains to thrombodefensins was reminiscent of the response to penicillin. Therefore, the response of these strains to penicillin in relation to the susceptibility to thrombodefensins was studied further (Chapter VI). Two penicillin-susceptible S.pneumoniae strains (R6 laboratory strains and a wild-type strain) showed lytic death on exposure to penicillin and thrombodefensins.

Penicillin and thrombodefensins induced both non-lytic death of cells of the penicillin-susceptible S.sanguis type II strain, but had a slight bactericidal effect with a lack of lysis on the genotypic penicillin-tolerant S.pneumoniae (Ly 4-4) strain and on S.sanguis strains. Viability of a penicillin-resistant S.sanguis type II strain was also slightly affected.

Penicillin pretreatment of thrombodefensins-susceptible strains blocked in part the bactericidal action of thrombodefensins. Competitive interaction between penicillin and thrombodefensins did not occur. We assume that penicillin-induced cell wall changes hindered thrombodefensins to reach the biologic target on the cytoplasmic membrane. The differences in susceptibility to thrombodefensins among streptococci may reflect altered cell wall synthesis in penicillin-tolerant and penicillin-resistant streptococcal strains. Another phenomenon noted in this chapter was that cells escaping the killing action of thrombodefensins, were even stimulated to grow. This effect was caused by other trypsin sensitive, heat-stable cationic platelet proteins.

Since penicillin-tolerant viridans group streptococci were resistant to the action of thrombodefensins, some studies were performed to improve the detection of penicillin-tolerant strains (Chapter VII and VIII). Penicillin susceptibility determinations made for strains of S.sanguis type II by two different broth dilution tests revealed small numbers of tolerant strains regardless of the volumes (0.01 and 0.1 ml) of subcultured broth. The addition of penicillinase to the subculture medium increased the number of tolerant strains significantly (Chapter VII).

In chapter VIII, we tested strains of nutritionally variant streptococci for their susceptibility to penicillin by a broth dilution method. All strains, except one were isolated from blood cultures of

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patients with bacterial endocarditis. All strains seemed to be susceptible, i.e. the minimal inhibitory concentrations were low. However, it was found that plates containing only vitamin B6 (pyridoxal HCl) and cysteine did not reveal true minimal bactericidal concentrations. When penicillinase was added to the subculture medium, all strains were found to have high minimal bactericidal concentrations and were known to be tolerant to penicillin.

Since the disk method is commonly used for susceptibility testing, we developed a disk method for demonstrating penicillin tolerance in viridans group streptococci. This was achieved by the substitution of the penicillin disk used for susceptibility testing after reading the zones of inhibition by a disk containing penicillinase. After reincubation penicillin-tolerant strains exhibited new growth in the area adjacent to this disk (Chapter IX).

The prevalence of penicillin tolerance among viridans group streptococci recovered from blood of patients with endocarditis and without endocarditis and isolated from the gingival sulcus was determined (Chapter X). In order to obtain indirect evidence that thrombodefensins may be relevant in the pathogenesis of endocarditis in human subjects, we determined the susceptibility to thrombodefensins as well. Among 73 isolates recovered from blood of patients with endocarditis 57 (78%) exhibited a tolerant response to penicillin. Seven (32%) of 22 isolates isolated from blood of patients without endocarditis, were penicillin-tolerant. The prevalence of penicillin tolerance among isolates obtained from gingival sulcus flora cultures taken from these patients and from patients with a cardiac disease at risk for endocarditis, was low: 65 (21%) of 314 strains were tolerant to penicillin. All S.sanguis type I isolates, except one appeared to be tolerant.

The degree of killing of viridans group streptococci by exposure to penicillin coincided with the degree of the reduction of the viable count by exposure to thrombodefensins. This indicates that in the patients with endocarditis caused by viridans group streptococci, selection may have occurred. This selection on these bacteria most likely was mediated by thrombodefensins. No further evidence to this hypothesis can be obtained from human studies. Therefore, we used the rabbit model to study the relevance of the susceptibility to thrombodefensins and

penicillin among S.sanguis type II strains in the pathogenesis of experimental endocarditis (Chapter XI). Two thrombodefensins-susceptible, penicillin-susceptible and two thrombodefensins-resistant, penicillin-tolerant strains were used in this investigation. The frequency of culture-positive VGs at 5 min after injection with a low number of bacteria was significantly higher after the challenge with the thrombodefensins-susceptible strains than after the challenge with the thrombodefensins-resistant strains. However, the frequency of endocarditis was the same for all these strains. No explanation so far can be given for this finding. In vitro studies demonstrated inactivation of the bactericidal activity of thrombodefensins when bound to various plastics. Further in vivo studies are necessary to elucidate the role of the catheter on the susceptibility of the rabbits to endocarditis.

To examine the significance of the susceptibility of S.sanguis type II strains to thrombodefensins further, we immunized rabbits with human thrombodefensins. These rabbits developed antibodies, which neutralized the bactericidal activity of rabbit and human thrombodefensins against the test organisms, as demonstrated by in vitro tests. The thrombodefensins-susceptible strain induced endocarditis significantly more often (60%) in immunized rabbits than in controls (21%; $p < 0.01$). Immunization did not influence the incidence of endocarditis by the thrombodefensins-resistant strain.

These findings support the hypothesis that platelets release thrombodefensins which kill susceptible strains among viridans group streptococci early after bacterial deposition at the vegetational surface.

The results of our investigations described in this thesis lead to the following fourteen conclusions:

- Two mechanisms appeared to be essential in the pathogenesis of experimental endocarditis caused by S.sanguis type II. One mechanism concerns the capacity of bacteria to adhere to cardiac vegetations. The other mechanism concerns the survival of adherent bacteria at that site.
- The survival of adherent bacteria was related to the susceptibility of the strains to platelet-released bactericidal substances.
- Platelet bactericidal substances called thrombodefensins most likely stored in the α -granules, were rapidly released by stimulation of

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- Thrombodefensins appeared to be low-molecular weight cationic proteins with antibacterial activity against a limited range of Gram-positive bacteria.
- In clots, a cooperative interaction exists between acid hydrolases (lysosomal enzymes) and thrombodefensins released from platelets.
- The response of pneumococci and viridans group streptococci to thrombodefensins was reminiscent to the response of these bacteria to penicillin. The degree of susceptibility to penicillin coincided with the degree of susceptibility to thrombodefensins.
- The bactericidal action of thrombodefensins on viridans group streptococci is enhanced by the presence of aminoglycoside antibiotics.
- The cell wall composition of penicillin-tolerant and penicillin-resistant viridans group streptococci and pneumococci is different from the cell wall composition of penicillin-susceptible viridans group streptococci and pneumococci
- Addition of penicillinase to the subculture medium used in broth dilution susceptibility testing improves the detection of penicillin-tolerant viridans group streptococci, including nutritionally variant strains.
- A disk-diffusion test substituting a penicillinase disk for a penicillin susceptibility disk, was a successful rapid screening test for the detection of penicillin-tolerant viridans group streptococci.
- Endocarditis due to viridans group streptococci is commonly caused by penicillin-tolerant, thrombodefensins-resistant strains.
- Prior to a dental procedure evaluation of the oral flora of patients with a cardiac disease at risk for endocarditis is not necessary, since penicillin-tolerant, thrombodefensins-resistant viridans group streptococci are commonly present in the gingival sulcus flora.
- The susceptibility of rabbits with pre-existing cardiac vegetations to endocarditis increased significantly after development of antibodies neutralizing the bactericidal activity of thrombodefensins.
- Preparation, isolation and purification of thrombodefensins are necessary in order to assess further the relevance of these cationic proteins in the innate host defense mechanism against intravascular infections.